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(57) Abstract

Methods, plants, and plant cells are provided for producing gene products to high levels. An *Agrobacterium* dual promoter derived form T-DNA sequences is used to initiate transcription. The dual promoter can be stimulated to high level expression by high levels of auxins. These plant hormones can be provided exogenously or by *in situ* means, using T-DNA genes to produce them in the plant cell.

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PLANT HORMONE REGULATED EXPRESSION OF GENETICALLY ENGINEERED GENE PRODUCTS IN PLANT CELLS

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Technical Field of the Invention

The invention relates to the production of gene products in plants and plant cells. More specifically the invention pertains to means of obtaining high level production of gene products in plants and plant cells.

BACKGROUND OF THE INVENTION

The T_R genes (right-most transferred genes) of some tumor-inducing (Ti) plasmids are frequently, but not always, found in plant tissues transformed by Agrobacterium tumefaciens. The T_R genes appear to be nonessential for oncogenesis. However, they do code for the biosynthesis of agropines, such as mannopine, which are secreted by infected plant cells and metabolized by A. tumefaciens.

Velten, et al, "T_R Genes Involved in Agropine Production," in Puhler, ed., Molecular Genetics of the Bacteria-Plant Interaction (1983), has shown that mutations in both the 1' and 2' genes of T_R abolish the ability to synthesize mannopine and agropine. (Hence, the genes are also referred to as mas genes.) Velten, et al., (EMBO Jour., Vol. 3, pp. 2723-30, 1984.) isolated a 479-bp DNA fragment containing the promoter for the 1' and 2' genes of T_R. The promoter fragment was fused in both orientations to the aph B gene of Tn5 (coding for neomycin and kanamycin resistance) and introduced into tobacco protoplasts. Calli were grown from the transformed protoplasts and APH(3')II enzyme activity was assayed. The 1' and 2' promoters were

found to express at roughly the same levels. The sequence of the fragment containing the 1' and 2' promoters was reported. Velten, et al., (Nucleic Acids Research, Vol. 13, pp. 6981-6998, 1985.) fused the T_R 1' and 2' promoter fragment to two different drug resistance genes, aph B and cat. The two genes were always found linked in transformed calli, even when only one drug resistance was selected. Harpster, et al., (Mol. Gen. Genet., Vol. 212, pp. 182-190, 1988,) linked the 1' and 2' promoters to octopine synthase and chitinase genes. The genes were expressed in transformed callus.

The T_R 1',2' promoter was found to express in plant protoplasts, callus, and whole plants transformed with plasmids containing the T_R 1',2' promoter linked to the lux A and lux B genes, respectively. (Koncz, et al., Proc. Natl. Acad. Sci. USA, Vol. 84, pp. 131-135, 1987.). Further, when transgenic plants were produced carrying the T_R 1',2' promoter linked to the aph B and Bacillus thuringiensis toxin (bt) genes, respectively, the level of insect resistance afforded by the bt gene in whole plants, was directly correlated with the level of kanamycin resistance afforded by the aph B gene. (Vaeck, et al., Nature, Vol. 328, pp. 33-37, 1987.)

The T_R 1' and 2' promoters linked to bacterial lac Z and aph B genes were found to be stimulated by wounding of plants. (Teeri, et al., EMBO Jour. Vol. 8, pp. 343-350, 1989.) Further, the dual promoter was found to be expressed to a much higher level (15 to 30-fold) in roots than in leaves.

However, up until now the stimulation of the T_R 1',2' dual promoter by specific plant hormones has not been described. Thus the full potential of the dual promoter as a means of producing large amounts of gene products efficiently in plants and plant cell culture has not been appreciated or exploited.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a method of producing a gene product in plant cells grown in culture.

It is another object of the invention to provide a method of producing a gene product in whole plant tissues.

It is yet another object of the invention to provide a method of producing a gene product in plant cells transformed with A. tumefaciens T-DNA.

It is still another object of the invention to provide a plant or plant cell for producing gene products.

These and other objects of the invention are provided by one or more of the embodiments described below. In one embodiment of the invention a method is provided for producing a gene product in plant cells, comprising: linking a gene coding for a gene product or for a protein which makes a gene product to A. tumefaciens mas 1',2' dual promoter to form an expression construct such that mRNA transcribed from the gene initiates at the mas 1',2' dual promoter; introducing the expression construct into a plant cell; growing progeny of the plant cell in the presence of about 10 to about 40 uM auxin; and harvesting the gene product from the progeny of the plant cell.

In another embodiment of the invention a method is provided of producing a gene product in tissues of a whole plant, comprising: linking a gene coding for a gene product or for a protein which makes a gene product to A. tumefaciens mas 1',2' dual promoter to form an expression construct such that mRNA transcribed from the gene initiates at the mas 1',2' dual promoter; introducing the expression construct into a plant cell; regenerating the plant cell to form a plant; harvesting the gene product from a tissue selected from the group consisting of: crown galls, crowns of the stem, and axillary buds.

In yet another embodiment of the invention a method is provided of producing a gene product in plant cells transformed with A. tumefaciens T-DNA, comprising: linking a gene coding for a gene product or for a protein which makes a gene product to A. tumefaciens mas 1',2' dual promoter to form an expression construct such that mRNA transcribed from the gene initiates at the mas 1',2' dual promoter; introducing the expression construct into a plant cell; introducing to the plant cell T-DNA of A. tumefaciens which encodes the synthesis of auxin to form a transformed cell; growing the transformed plant cell; harvesting the gene product from the transformed plant cell or its progeny.

In still other embodiments of the invention plants and plant cells are provided. comprising a gene coding for a gene product or for a protein which makes a gene product linked to A. tumefaciens mas 1',2' dual promoter such that mRNA transcribed from the gene initiates at the mas 1',2' dual promoter; and T-DNA of A. tumefaciens which encodes the synthesis of auxin.

These and other embodiments which are detailed below provide the art with means to greatly overproduce gene products in plant cells. This provides a distinct economic benefit by allowing a higher yield of gene product per plant or plant cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows hybridization of lux A . lux B and aph B probes to poly (A)+ RNA prepared from leaves of transformed tobacco plants containing the mas 1',2' promoter-luciferase fusion. The numbers at the left of each lane indicate the size of the hybridizing RNA in bases.

Figure 2 depicts low-light video-image analysis of mas promoter activity in plant organs using the bacterial luciferase reporter enzyme. Cell photographs were prepared by superimposition of the bioluminescent image upon the video image of the plant organ. Color calibration bar from bottom to top indicates increasing numbers of photons. (A) Expression of mas promoter luciferase A and B gene fusion in stem internode sections of flowering (bottom) and nonflowering (middle) transgenic tobacco plants. Stem sections from an untransformed tobacco plant are also shown (top). Stem sections from one plant are arranged horizontally from left to right from the base to the shoot apex. (B) Activity of the mas promoters in leaf tissues. The lower left and right leaves correspond to the tenth and fourth leaves, respectively, below the vegetative shoot apex of a 30-cm-tall transgenic plant. The top leaf (horizontal) is taken from an untransformed tobacco plant. (C) Activity of the mas promoters in sepal, stigma, and petals of the corolla of transgenic tobacco plant flower (left), longitudinal section through flower from transformed plant (middle), and a flower from an untransformed plant (right) are shown. (D) Activation of axillary buds 12 hr after apical meristem

removal. One of two 40-cm-tall identical transgenic tobacco plants was decapitated. After incubation of both plants at room temperature for 12 hr, an 8-cm stem segment was removed from the top of the decapitated plant (right) and the intact plant (left) and sliced along the longitudinal axis. The halved stem segments were placed side by side in a tissue culture dish, and bioluminescence was measured for 1 hr by low-light video-image analysis. (E) Inhibition of the activity of the mas promoters. (inset left) Stem section excised from the ninth internode below the shoot apex of a 60-cm-tall nonflowering plant incubated for 12 hr on filter paper saturated with 5 uM naphthalene acetic acid (NAA). (inset right) Internodal segment of spiral stem (3 cm) excised from the same region placed on the upper surface of a stem section. Inhibition of luciferase activity in serial stem sections was measured by video-image analysis. (Upper and lower left) Sections were treated with auxin only. (Upper and lower right) Stem sections were covered for 12 hr with stem segments prior to low-light video analysis. Curve (in red) at base of the panel indicates the distribution of photons detected in the area delineated by the horizontal blue lines. (F) Activity of the mas 1',2' dual promoter in wild-type A. tumefaciens stem tumors induced on transgenic tobacco plants. (Upper) From left to right: stem section from an untransformed plant; section excised 1 cm above stem tumor on a transgenic plant; section through the center of stem tumor; and section excised 1 cm below stem tumor. All tissue sections (upper) were measured by low-light video-image analysis immediately after excision from the stem, only the tumor tissue emits light. (Lower) Stem sections are identical to the upper row with respect to their position in the tumorous transgenic plant. As a positive control, luciferase activity was measured after 12 hr incubation of the sections in 5 uM NAA.

Figure 3 shows the influence of auxin and cytokinin on mas promoter activity in: (A) stem sections and (B) leaf discs. Luciferase activity was measured by luminometric assay.

DETAILED DESCRIPTION OF THE INVENTION

It is a finding of the present invention that the mannopine synthase dual promoter of Agrobacterium tumefaciens is stimulated by auxin and cytokinin. The level of stimulation is dramatic; under some conditions expression from the promoter is increased about 100-fold. This sensitivity to plant hormones allows the overproduction of gene products which are under the control of the mannopine synthase (mas) dual promoter. This finding leads to the use of the dual promoter (also called the mas 1',2' promoter and the T_R 1',2' promoter) in a number of different embodiments, each of which will be described below.

In the context of this specification, the term gene product will be used to encompass both a protein which is the encoded product of a particular gene, as well as a product which is made by the protein product of a particular gene. Thus, for example, octopine synthase would be a gene product by virtue of being a protein encoded by the octopine synthase genes, and octopine itself would be a gene product in that it is produced by the proteins encoded by the octopine synthase genes. Genes coding for gene products can be from any source: plant, bacterial, or animal. However, when in the context of the present invention a gene is referred to as being linked to the mas 1', 2' dual promoter, the mannopine synthase genes which are naturally found under the control of the mas 1', 2' dual promoter are expressly excluded. Some particularly preferred plant gene products include alkaloids, such as sanguinarine. Particularly preferred bacterial gene products include antibiotics. Particularly preferred animal gene products include somatotropin, oxytocin, and insulin. Regardless of the species or genus source of the gene linked to the mas dual promoter, enhanced expression can be achieved according to the methods of the present invention. If the gene product desired is the end product of a complex metabolic pathway involving many enzymes, it may be desirable to link the genes coding for enzymes catalyzing rate limiting steps or steps at branch-points of the pathway to the mas dual promoter to enhance efficiency of end product production.

All of the embodiments of the present invention involve linking a gene coding for a gene product (or for protein which makes a gene product) to the A. tumefaciens mas 1',2' dual promoter. The linkage is such that an expression construct is formed in which mRNA transcribed from the gene initiates and is regulated at the mas 1',2' dual promoter. Such expression constructs are known and can be formed as described in Velten, et al., EMBO Journal, supra; Velten, et al., Nucleic Acid Research, supra; Harpster, et al., Molecular and General Genetics, supra; Koncz, Proc. Natl. Acad. Sci., USA, supra; Vaeck, et al., Nature, supra; and Teeri, et al., EMBO Journal, supra. The mas 1',2' dual promoter can be isolated on a fragment of less than 500 base pairs and fused by techniques known in the art to genes of bacterial, plant, or animal origin, as discussed above. The mas 1',2' dual promoter is called a dual promoter because it has back-to-back promoters which direct transcription in opposite directions. Thus the dual promoter provides spaces for the insertion of two genes or gene cassettes.

According to the present invention a gene encoding a gene product which is desired can be linked to either the mas 1' or the mas 2' side of the dual promoter. Also in accordance with the present invention the same gene coding for a gene product can be linked to both sides of the dual promoter. If only one of the two sides of the dual promoters is used for the gene coding for the gene product, then it is desirable to place a so-called reporter gene on the other side of the dual promoter. A reporter gene comprises any gene which can be easily and conveniently monitored for expression. Drug resistance genes are often used for this purpose, for example the chloramphenicol acetyl transferase gene (cat) or the neomycin phosphotransferase gene (aph B). Particularly preferred in the practice of the present invention as a reporter gene is a fusion of the bacterial luciferase genes of Vibrio harvevi. Fusion of the luciferase (lux A and lux B) genes has been accomplished and taught by Olsson, Mol. Gen. Genet., Vol. 215, pp. 1-9, 1988. The fusion products are functional and their photon products can be readily observed both in vivo in whole plants and in vitro in cell-free systems.

The expression construct of the present invention which comprises a gene coding for a gene product or for protein which makes a gene product linked to the A. tumefaciens mas 1',2' promoter, can be introduced to plant cells using any of the many techniques known in the art. For example, transformation can be accomplished by any of the chemical or physical techniques known, including electroporation, microinjection, particle gun, laser, calcium phosphate and polyethylene glycol, and calcium nitrate and polyethylene glycol. Plant cells may also receive the expression construct by means of protoplast fusion, wherein plant cells and the cells carrying the expression construct, usually bacterial cells, are both made into protoplasts which are fused according to techniques well known in the art. Yet another means of introducing the expression construct of plants cells involves co-cultivation of plant cells with Agrobacterium tumefaciens as well as bacteria carrying the expression construct. According to this technique a genetic element in the Agrobacterium mobilizes the expression construct from the bacterial cells into the plant cells. As will be clear to those of skill in the art, the means of introduction of the expression construct into plant cells is not critical to the invention.

Plant cells according to the present invention include cells of plants of all types. Both wild and cultivated plants may be employed. Both monocots and dicots are contemplated.

Plant cells which have been transformed with the expression construct can be grown in the presence of high concentrations of an auxin in order to induce the expression of the gene product which is linked to the mas dual promoter. The auxin may be a natural or a synthetic compound, as is known in the art. Concentrations of auxin are between about 10 uM and about 40 uM, preferably greater than about 25 uM. Cytokinin may also be added to the growth medium of the cells at concentrations ranging from about 0.5 uM to about 10 uM. Again the cytokinin may be a natural or synthetic product. It is desirable that the ratio of auxin to cytokinin be high in order to achieve maximal stimulation of the dual promoter. The plant cell and its progeny may be grown in cell suspension culture, or as callus in

culture. Both types of cultures produce ample quantities of the gene product. Media and techniques for growing plant cells as suspension or callus in culture are well-known in the art. It may be desirable before growing cells in culture that the transformed cells carrying the expression construct be regenerated into plants or plantlets. By regeneration one ensures that the cells which one grows and cultures are derived from a single progenitor cell.

Harvesting of gene products from plant cells may be accomplished according to any means known in the art. Under some conditions gene products may be secreted from the cells and then harvested directly from the culture media. However, more typically gene products will be harvested by collecting the plant cells grown in the presence of auxin and extracting the gene product from the plant cells, according to techniques known in the art. Again the means of extraction of the gene product from the plant cells is not critical to the invention.

In another embodiment of the present invention the gene product is not harvested from cell cultures but is harvested from tissues of plants. According to this embodiment the expression construct is again introduced into plant cells as described above and transformed plant cells are regenerated to form whole plants, according to techniques well known in the art. It is a finding of the present invention that expression from the mas 1',2' dual promoter is extremely tissue-specific. Certain tissues have been found to express proteins from the dual promoter at very high levels, presumably because the plant hormone environment is proper for high level expression from the dual promoter. The tissues which have been found to foster such high level expression from the dual promoter include the crown of the stem, (i.e., the portion of the stem immediately below the surface of the soil,) the axillary buds, either after the shoot apex has been removed or after the plant has flowered, and crown gall tissue. Crown galls can be formed by infection with Agrobacterium, for example. The gene product can be harvested from these tissues which are particularly good expressers of the dual promoter, according to any means known in the art. Again, means for extracting

proteins and gene products from plant tissues are well known and are not critical to the practice of the present invention.

It may be desirable in the case of crown gall tissue rather than extracting the gene product directly from the tissue harvested from the plant, to grow cells of the crown gall (which carry both T-DNA as well as the expression construct) axenically in culture before harvesting the gene product. Such methods are well known in the art. The T-DNA of Agrobacterium tumefaciens is the portion of the bacterial DNA which is transferred and integrated into the plant nuclear genome. The reason why crown gall tissue is thought to express proteins from the dual promoter so highly is because the T-DNA carries the genes for both auxin and cytokinin. Thus the crown gall tissue makes its own plant hormones in excess of those found in surrounding stem tissue. Growth of such plant cells (containing both the expression construct and T-DNA) in culture obviates the need to add exogenous auxin to the culture medium to achieve enhanced expression from the dual promoter.

In another embodiment of the present invention the expression construct of the present invention is introduced into a plant cell as well as introducing T-DNA of Agrobacterium tumefaciens into the same plant cell. The means of introduction of both the T-DNA and the expression construct may be any that is known in the art. As already discussed above, these include chemical and physical means including electroporation, protoplast fusion, co-cultivation, infection, and transformation. The plant cell which contains both the expression construct and the T-DNA encoding auxin synthesis may be grown in cell culture as a callus or as a cell suspension. However, because of the presence of the T-DNA genes encoding the plant hormone synthesis, no exogenous auxin need be added to induce high level expression from the dual promoter, as the plant cells will make their own. The gene product can be harvested from the plant cells or plant cell culture medium, as discussed above, according to any means known in the art. The T-DNA may also carry the genes for cytokinin production.

Also provided by the present invention are plant cells which contain both the expression construct of the present invention as well as T-DNA encoding auxin synthesis. As described above, these can be introduced into the plant cell according to any means known in the art. Such plant cells are useful for growth in cell culture to produce abundant quantities of the gene product linked to the dual promoter. The cells may also carry the T-DNA gene for cytokinin production.

Also provided by the present invention are plants in which at least some of the cells of the plant contain both the expression construct of the present invention, i.e., the mas dual promoter linked to a gene coding for a gene product, as well as T-DNA encoding auxin synthesis. Such plants, can be made, for example by transformation of a plant cell with the expression construct and subsequent regeneration into a plant. The plant can then be infected with Agrobacterium, for example on its stem, such that some of the stem cells of the plant become carriers of both the expression construct and the T-DNA encoding auxin synthesis. Other means for making such plants may be used and are within the scope of the present invention. The plants may also carry the T-DNA gene for cytokinin synthesis.

The following experimental results are provided to exemplify certain aspects of the present invention, but do not limit the scope of the invention which has been described above.

Example 1

This example demonstrates that equal amounts of transcripts are synthesized from the mas 1' and 2' promoters in transformed plant tissues.

The lux A and lux B genes encoding a heterodimeric luciferase enzyme in Vibrio harveyi were converted to structural gene cassettes, linked to the mas 1',2' dual promoters in the plant expression vector pPCV701 lux A&B, and transformed into tobacco plants. (Koncz, et al., PNAS, Vol. 84, pp. 131-135, 1987).

For Northern RNA hybridization analysis poly(A)+ RNA was prepared, separated on formaldehyde gels, and blotted onto nitrocellulose filters.

Hybridization was performed between poly(A)+ RNA (20 ug) prepared from leaves of transformed tobacco plants containing the mas promoter luciferase fusion and the Sal I lux A DNA fragment A and the Bum HI lux B DNA fragment B of pPCV701 lux A&B DNA. Hybridization of APH (3')-II DNA probe isolated as Bcl I-Bam HI fragment from plasmid pPCV002 DNA (Koncz, et al., Mol. Gen. Genet., Vol. 204, pp. 383-396, 1986), to the plant poly(A)+ RNA sample is shown. Identical amounts of DNA fragments were labeled and probes with similar specific activities were used for hybridization.

Quantitative transcript analysis demonstrated that equal amounts of lux A and lux B transcripts were synthesized from the mas 1' and 2' promoters in transformed plant tissues (Fig. 1), indicating that sequences located in a 200-base-pair region between the 1' and 2' promoters, regulate the bidirectional transcription.

Plasmid pTB7, Baldwin, et al., Biochemistry, Vol. 23, pp. 3663-67, 1984) carrying the luxAB transcriptional unit, was linearized with Sal I and treated with various amounts of BAL-31 exonuclease. lux A fragments isolated from gels after EcoRI digestion were subcloned into the Dra I-EcoRI sites of pBR322. The ATG codon preceding the translation initiation codon of luxA is part of an AAA triplet. Regeneration of Dra I sites indicated that the endpoints of deletions were AAA triplets. The DraI-EcoRI fragment pool obtained from pBR322 recombinants was cloned into Sma I-EcoRI sites of the M13 vector mp18, and the exact endpoints of deletions were determined for 196 independent clones by DNA sequencing. One of the isolated deletion endpoints was located 7 bp upstream from the ATG initiation codon of luxA. This plasmid was opened at its BamHI site, treated with Bal-31 to make further deletions, and then religated. Out of 98 clones sequenced, the extra ATG was removed from 8, and of these, 4 retained the Sal I site of mp18. M13 replicative form DNA was isolated from one of these clones, digested with EcoR1, treated with mung bean nuclease, and ligated to Sal I linkers. The resulting luxA gene Sal I fragment, with all extra ATG codons removed, was sequenced in both directions.

The luxB gene was isolated as an Ssp I-Pvu II DNA fragment from the plasmid pTB7; following addition of BamHI linkers to the 5' and the 3' ends, the fragment was inserted in both orientations into the BamHI site of M13 mp18 and partially sequenced. The Sal I lux A cassette and the BamHI lux B cassette were inserted in two steps into single Sal I and BamHI sites of pPCV701, respectively This resulted in plasmid pPCV701lux A&B and rendered lux A under gene 1' and luxB under gene 2' promoter control.

Example 2

This example demonstrates that the amount of luciferasee activity in plants transformed with mas 1'.2' dual promoter-luciferase constructs varies with the organ or tissue type.

Nicotiana tabacum cv Petit Havana SR1 leaf discs (7 mm) were infected with A. tumefaciens strain GV3101 pMP904K containing the bacterial luciferase plant expression vector pPCV-701 lux A&B. The leaf discs were transferred to MS medium containing NAA (0.1 mg/liter), BAP (0.5 mg/liter), kanamycin (100 mg/liter), and claforan (400 mg/liter) for selection of transformants. Plants were regenerated from antibiotic-resistant calli. Luciferase activity was measured in homogenate of callus, stem, and root tissues of 20 1-month-old 2-cm-tall plantlets and from flowering tobacco plants, (1 meter tall) grown from the seed of self-pollinated N. tabacum SR1 plants. Luciferase activity in leaf and corolla tissue was calculated based on the average L.U. detected in three tissue discs (7 mm) excised with a paper punch from a leaf two modes above the base of the plant or from a flow. Luciferase activity in stem intermode sections was based on the average L.U. detected in homogenates from four consecutive sections taken from the ninth internode below the shoot apex. Results are shown below in Table 1.

Table 1. Differential expression of mas promoter driven luciferase reporter genes in organs and tissues of transgenic tobacco plants

Organ/tissue	Luciferase activity L.U./ug of protein	Organ/tissue	Luciferase activity L.U./ug of protein
Callus	63.3	Flower (corolla)	0.14
Plantlet		Petal	
Shoot	0.04	Tip	5.4
Root	7.9	Middle	1.2
Leaf (stem location)		Base	0.6
Top	0.06	Sepal	0.24
Middle	0.10	Stamen	0.37
Bottom	1.3	Anther	0.7
Leaf (basal)		Filament	0.6
Tip	1.7	Pollen	
Middle	0.6	Germinated	22.2
Base	0.3	Ungerminated	0.0
Stem (internodes)		Pistil	0.82
Top (2nd)	0.12	Stigma	9.4
Middle (6th)	0.35	Style	1.2
Bottom (13th)	1.27	Ovary	0.1
Stem section			
Epidermis	0.12		
Vascular tissue	1.31		
Pith	0.33		
Root tip	51.7		

Calli maintained at a high auxin to cytokinin ratios displayed 200-fold higher activities than differentiated plant tissues.

At low auxin to cytokinin ratios, calli formed shoots, and the activity of the mas promoters decreased.

In seed-derived plantlets, luciferase was expressed at the crown of the stem (i.e., the region of the stem below the soil level) at much higher levels than in other organs. Shoot tips of soil-grown plants displayed the lowest activity when compared with other tissues. In stems, leaves, and petioles of nonflowering plants, a gradual increase in luciferase activity was observed from the shoot apex toward the base. In the stem, maximum luciferase activities were located in the cambium and vascular tissues. This result may reflect the high density of cells in vascular tissues.

Leaves displayed a gradient of bioluminescence, resulting in a 30-fold increase in luciferase expression from the leaf base to the tip (Fig. 2B).

During flowering, the basipetal expression gradient disappeared resulting in an increased and similar level of luciferase expression throughout all stem and leaf tissues examined. In flowers, 2 days prior to opening, a dramatic increase of luciferase activity was detected in nonfused portions of the corolla. A basipetal expression gradient, was also found in the sepal, petal, and pistil (Fig. 2C). The mas promoters were silent in pollen, but became highly active within the first hour of pollen germination.

The expression of mas promoter luciferase gene fusions was compared with the nopaline synthase promoter-APH(3')-II gene fusion contained in pPCV701 lux A&B T-DNA, as internal standard. Tissues extracts were prepared as described (Velten, et al., EMBO Journal, Vol. 3, pp. 2723-2730, 1984) and assayed for APH(3')-II activity using kanamycin sulfate and (gamma ^{32}P)ATP substrates (Koncz, et al., Mol. Gen. Genet., Vol. 294, pp. 383-396, 1986). In samples containing identical amounts of protein, the relative activity of APH(3')-II enzyme was determined by densitometric scanning of kanamycin phosphate spots on autoradiograms obtained by APH(3')-II gel assays.

Although displaying some variability in plant tissues, the distribution of nopaline synthase promoter driven APH(3')-II gene activity differed in pattern and level of expression from the described activity of the mas promoter.

Example 3

This example demonstrates that the activity of the mas 1',2' dual promoter is stimulated by auxin and by wounding.

The shoot apex and leaf primordia are known centers of auxin synthesis and their removal temporarily arrests polar auxin transport in vegetative seed plants. To test the correlation between an auxin gradient in the plant and the relative activity of the 1',2' dual mas promoters, nonflowering 1.0-meter-tall transgenic tobacco plants were decapitated in the middle of the tenth internode below the shoot

apex and the stem was encased in a Tygon™ tubing sleeve to form a wall. The cut stem surface was treated with water or 10 uM NAA.

NAA treatment resulted in a 130-fold increase in reporter enzyme activity when compared to stem samples taken immediately after removing the shoot apex (Table 2).

Table 2. Wound and auxin-mediated activation of mas promoters in stem sections from decapitated transgenic plants

Time, hr	Auxin	Luciferase activity L.U./ug of protein	Increase of activity L.U./ug of protein	APH(3')-II units/ug of protein	Increase of activity APH(3')-II units/ug of protein
0	-	0.5	1	3.0	1
24	-	24.0	48	8.5	3
72	-	33.0	66	-	-
0	+	1.4	1	4.5	1
24	+	187.0	134	16.4	3.6
72	+	204.0	146	6.1	1.4

Activity of the luciferase reporter enzyme also increased about 50-fold in untreated stem sections, indicating a wound-induced activation of the mas promoters. Since the activity of the nopaline synthase promoter-driven APH(3')-II gene increased only 3-fold, we conclude that extracellular addition of auxin enhances expression of the mas 1',2' dual promoter-luciferase gene fusion.

Example 4

This example demonstrates the stimulatory effects of plant hormones on the expression of the A. tumefaciens mas 1',2' dual promoter in stem sections and leaf discs.

Stem sections were excised 10-12 internodes below the shoot apex of nonflowering, plants which had been transformed with the mas 1',2' dual promoter linked to the lux A and lux B genes. The sections were incubated on filter paper discs saturated with: (a) water; (b) 1 uM benzylaminopurine (BAP); (c) 10 uM naphthaleneacetic acid (NAA); (d) 1 uM BAP and 10 uM NAA; or (e) 1 uM BAP, 10 um NAA and 5 ug/1 ml cyclohexamide. At selected time intervals stem slices were homogenized and assayed for luciferase activity as described below.

As can be seen in Figure 3A, auxin (NAA) increased the wound-induced stimulation of luciferase. Cytokinin alone (BAP) stimulated, although less than auxin. Mixtures of auxin and cytokinin stimulated similarly to auxin alone. All stimulation, including wound-induced, was abolished by the protein synthesis inhibitor cyclohexamide.

Leaf discs (7 mm) of young, fully expanded leaves were excised and incubated for selected time intervals on filter paper. The filter papers were saturated with: (a) 0.3 uM BAP; (b) 0.3 uM BAP and 0.5 uM NAA; (c) 0.3 uM BAP and 5 uM NAA; (d) 0.3 uM BAP and 40 uM NAA. Results are shown in Figure 3B. Over 25 hr, a continuous increase of light production was detected that correlated with auxin concentration and that reached maximum activity 4-5 days after incubation of the leaf discs in the MS plant culture medium.

Luciferase assays were performed as follows. Plant tissues [40-60 mg (fresh weight)] were homogenized in 1.0 ml of luciferase reaction buffer (50 uM sodium phosphate, pH 7.0/50 uM 2-mercaptoathanol/0.4 M sucrose) and cleared by centrifugation in an Eppendorf centrifuge for 5 min at 4°C. After determination of protein concentration, the extracts were supplemented with 0.1% bovine serum albumin and aliquots were assayed for luciferase activity in a luminometer. The luminometric measurements were standardized with defined amounts of purified luciferase light emission standard. One light unit (L.U.) is equivalent to 1.6×10^6 photons per sec.

Induction of the mas promoters in stem sections and leaf discs may be due to wound-induced ethylene production. However, treatment of stem sections with the ethylene-generating compound chloroethyl phosphoric acid (10 ug/ml) or with ethylene inhibitors, e.g., cobalt chloride (0.1 uM) and aminovinylglycine (0.1 uM) did not enhance or inhibit luciferase expression, when added alone or with 10 uM NAA. In contrast, stem sections incubated with the auxin inhibitor 1,3,5-triiodobenzoic acid applied at concentrations of 1 uM to 1 mM resulted in a 10-99% inhibition of mas promoter activity.

Example 5

This example shows that the apical meristem contains a factor that inhibits auxin-induced stimulation of mas 1',2' dual promoter activity.

Shoot segments (3 cm long), derived from the stem apex, were applied to the upper surface of stem sections (2 uM) on filter paper saturated with 5 uM NAA. Almost complete inhibition of luciferase expression in the stem discs was observed 12 hr after application of the stem segment (see Fig. 2D). This result indicates that the shoot apex produces an inhibitory substance that is probably transported basipetally in the stem and that down-regulates the dual mas promoters by counteracting auxin stimulation.

Within 12 hr after removal of the shoot apex in nonflowering plants, the light emission of axillary buds increased dramatically (Fig. 2E). This result indicates that either auxin concentration is increased in axillary buds or that an inhibitor is removed in the absence of apical dominance.

Treatments of auxin-activated stem sections with 10 uM to 1 mM abcisic acid (ABA) resulted in a 22-67% inhibition of mas promoter activity, respectively. Whether the physiological inhibitor is identical to ABA or to other auxin induced ABA-like compounds proposed to balance auxin action in stems and leaves remains to be determined.

Example 6

This example shows that there is an inhibitor of mas 1',2' dual promoter expression in leaf tissues.

Leaf tissues were converted to protoplasts. This conversion resulted in a 500-fold increase in mas promoter activity, independent of hormone concentration, during protoplast isolation. When protoplast cultures were allowed to form calli, luciferase activity was similar to that detected in calli derived from organ explants. The extent of mas promoter activation due to protoplast formation clearly exceeded the wound-induced response, indicating that an inhibitor was removed from leaf tissues by the protoplast isolation procedure.

Example 7

This example demonstrates that other T-DNA genes of A. tumefaciens influence the activity of the mas 1'.2' dual promoters.

Tumors were induced on stems of transgenic tobacco plants with wild-type and mutant A. tumefaciens strains carrying deletions of either iaaM, iaaH, or ipt genes which specify the intracellular synthesis of auxin and cytokinin in the T-DNA of the Ti plasmid B6S3 (Garfinkle, et al., Cell, vol. 27, pp. 143-153, 1981).

Luciferase activity was measured in combined tissue extract of four tumor slices. In experiments in which the stem was inoculated with mutant strains, luciferase activities were determined in stem sections containing the inoculation site.

Wild-type tumors, 1-month-old, expressed the luciferase reporter enzyme at about 150-fold higher levels than those detected in stem sections above or below the tumor (Fig. 2F). In the absence of iaaM or iaaH genes, luciferase levels at infection sites were identical to those found in uninfected stem sections. Deletion of the ipt gene resulted in a decrease in luciferase activity to about 50% of that found in wild-type tumors. (See Table 3.) These results indicate that the activity of the mas promoters is regulated by the ratio of auxin to cytokinin in tumor tissues.

Table 3. iaaM and iaaH genes regulate mas promoters in tumors.

Agrobacterium strain	Mutation in T-DNA	Luciferase activity, L.U./ug of protein
LD-1		149
A6#328	iaaM	13
A6#393	iaaH	2
A6#338	ipt	69

Example 8

This example demonstrates that the mas 1', 2' dual promoter linked to the tyrosine decarboxylase gene when placed in cells of Papaver somniferum (L.) can be used to produce the benzophenanthridine alkaloid sanguinarine.

Callus cultures are produced from seedling parts of Papaver somniferum on plant growth medium containing 2,4-D (2,4-dichlorophenoxy acetic acid) at a concentration of 1 mg/liter of medium. This callus (or cell suspension derived from the callus) are transformed with constructs of the mas 1',2' dual promoter linked to the tyrosine decarboxylase gene isolated from Papaver somniferum or other sources. The tyrosine decarboxylase gene is the first enzyme in the benzophenanthridine pathway, taking the amino acid tyrosine to tyramine. This commits the tyrosine pool to the pathway, the end product of which is sanguinarine. The action of the promoter expresses the gene at high levels in the presence of auxin, thus increasing the flow of the precursor into the pathway, thereby increasing yield.

Any of the genes for the other sixteen enzymes of the benzophenanthridine pathway can be linked together with tyrosine decarboxylase or in other combinations with the mas 1',2' dual promoter to effect an increase in the production of sanguinarine or other benzophenanthridine alkaloids.

CLAIMS

1. A method of producing a gene product in plant cells, comprising:

linking a gene coding for a gene product or for a protein which makes a gene product to A. tumefaciens mas 1',2' dual promoter to form an expression construct such that mRNA transcribed from the gene initiates at the mas 1',2' dual promoter;

introducing the expression construct into a plant cell;

growing progeny of the plant cell in the presence of about 10 to about 40 uM auxin; and

harvesting the gene product from the progeny of the plant cell.

2. The method of claim 1 wherein the plant cell is grown in culture as a callus.

3. The method of claim 1 wherein the plant cell is grown in culture as a cell suspension.

4. The method of claim 1 wherein after introducing the expression construct into a plant cell, the plant cell is regenerated into a plant, and wherein the progeny of the plant cell growing in the presence of about 10 to about 40 uM auxin is derived from tissue of the plant.

5. A method of producing a gene product in plant cells comprising:

linking a gene coding for a gene product or for a protein which makes a gene product to A. tumefaciens mas 1',2' dual promoter to form an expression construct such that mRNA transcribed from the gene initiates at the mas 1',2' dual promoter;

introducing the expression construct into a plant cell;

regenerating the plant cell to form a plant;

harvesting the gene product from a tissue selected from the group consisting of: crown galls, crowns of stem, and axillary buds.

6. The method of claim 5 wherein the axillary buds are collected after a shoot apex has been removed from the plant or after the plant has flowered.

7. The method of claim 5 wherein the tissue is a crown gall which is formed by infecting the plant with A. tumefaciens.

8. The method of claim 7 wherein prior to harvesting the gene product, cells of the crown gall are grown axenically in culture.

9. A method of producing a gene product in plant cells, comprising:

linking a gene coding for a gene product or for a protein which makes a gene product to A. tumefaciens mas 1',2' dual promoter to form an expression construct such that mRNA transcribed from the gene initiates at the mas 1',2' dual promoter;

introducing the expression construct into a plant cell;

introducing to the plant cell T-DNA of A. tumefaciens which encodes the synthesis of auxin to form a transformed cell;

growing the transformed plant cell in culture;

harvesting the gene product from the transformed plant cell or its progeny.

10. The method of claim 9 wherein the T-DNA is introduced by electroporation.

11. The method of claim 9 wherein the T-DNA is introduced by transformation.

12. The method of claim 9 wherein the T-DNA is introduced by protoplast fusion of an A. tumefaciens and a plant cell.

13. The method of claim 9 wherein the T-DNA is introduced by infection of the plant cell with A. tumefaciens.

14. The method of claim 1 wherein the expression construct is introduced by electroporation.

15. The method of claim 1 wherein the expression construct is introduced by transformation.

16. The method of claim 1 wherein the expression construct is introduced by infection of the plant cell with A. tumefaciens.

17. The method of claim 5 wherein the expression construct is introduced by electroporation.

18. The method of claim 5 wherein the expression construct is introduced by transformation.

19. The method of claim 5 wherein the expression construct is introduced by infection of the plant cell with A. tumefaciens.

20. The method of claim 9 wherein the expression construct is introduced by electroporation.

21. The method of claim 9 wherein the expression construct is introduced by transformation.

22. The method of claim 9 wherein the expression construct is introduced by infection of the plant cell with A. tumefaciens.

23. The method of claim 1 wherein the gene is linked to the mas 1' promoter.

24. The method of claim 1 wherein the gene is linked to the mas 2' promoter.

25. The method of claim 1 wherein a copy of the gene is linked to both the mas 1' and 2' promoters.

26. The method of claim 23 wherein the mas 2' promoter is linked to a reporter gene.

27. The method of claim 24 wherein the mas 1' promoter is linked to a reporter gene.

28. The method of claim 26 wherein the reporter gene is a fusion of the lux A and lux B genes of Vibrio harvevi.

29. The method of claim 27 wherein the reporter gene is a fusion of the lux A and lux B genes of Vibrio harvevi.

30. The method of claim 5 wherein the gene is linked to the mas 1' promoter.

31. The method of claim 5 wherein the gene is linked to the mas 2' promoter.

32. The method of claim 5 wherein a copy of the gene is linked to both the mas 1' and 2' promoters.

33. The method of claim 30 wherein the mas 2' promoter is linked to a reporter gene.

34. The method of claim 31 wherein the mas 1' promoter is linked to a reporter gene.

35. The method of claim 33 wherein the reporter gene is a fusion of the lux A and lux B genes of Vibrio harvevi.

36. The method of claim 34 wherein the reporter gene is a fusion of the lux A and lux B genes of Vibrio harveyi.

37. The method of claim 9 wherein the gene is linked to the mas 1' promoter.

38. The method of claim 9 wherein the gene is linked to the mas 2' promoter.

39. The method of claim 9 wherein a copy of the gene is linked to both the mas 1' and 2' promoters.

40. The method of claim 37 wherein the mas 2' promoter is linked to a reporter gene.

41. The method of claim 38 wherein the mas 1' promoter is linked to a reporter gene.

42. The method of claim 40 wherein the reporter gene is a fusion of the lux A and lux B genes of Vibrio harveyi.

43. The method of claim 41 wherein the reporter gene is a fusion of the lux A and lux B genes of Vibrio harveyi.

44. A plant cell, comprising:

a gene coding for a gene product or for a protein which makes a gene product, linked to A. tumefaciens mas 1',2' dual promoter such that mRNA transcribed from the gene initiates at the mas 1',2' dual promoter; and

T-DNA of A. tumefaciens which encodes the synthesis of auxin.

45. A plant wherein at least some of its cells, comprise:

a gene coding for a gene product or for a protein which makes a gene product linked to A. tumefaciens mas 1',2' dual promoter such that mRNA transcribed from the gene initiates at the mas 1',2' dual promoter; and

T-DNA of A. tumefaciens which encodes the synthesis of auxin.

46. The plant cell of claim 44 wherein the gene is linked to the mas 1' promoter.

47. The plant cell of claim 44 wherein the gene is linked to the mas 2' promoter.

48. The plant cell of claim 44 wherein a copy of the gene is linked to both the mas 1' and 2' promoters.

49. The plant cell of claim 46 wherein the mas 2' promoter is linked to a reporter gene.

50. The plant cell of claim 47 wherein the mas 1' promoter is linked to a reporter gene.

51. The plant cell of claim 49 wherein the reporter gene is a fusion of the lux A and lux B genes of Vibrio harveyi.

52. The plant cell of claim 50 wherein the reporter gene is a fusion of the lux A and lux B genes of Vibrio harveyi.

53. The plant of claim 45 wherein the gene is linked to the mas 1' promoter.

54. The plant of claim 45 wherein the gene is linked to the mas 2' promoter.

55. The plant of claim 45 wherein a copy of the gene is linked to both the mas 1' and 2' promoters.

56. The plant of claim 53 wherein the mas 2' promoter is linked to a reporter gene.

57. The plant of claim 54 wherein the mas 1' promoter is linked to a reporter gene.

58. The plant of claim 56 wherein the reporter gene fusion of the lux A and lux B genes of Vibrio harveyi.

59. The plant of claim 57 wherein the reporter gene is a fusion of the lux A and lux B genes of Vibrio harveyi.

60. A method of producing a gene product in plant cells, comprising:

growing a plant cell in the presence of about 10 to about 40 uM auxin, said plant cell comprising a gene coding for a gene product or for a protein which makes a gene product, linked to A. tumefaciens mas 1',2' dual promoter such that mRNA transcribed from the gene initiates at the mas 1',2' dual promoter; and

harvesting the gene product from the progeny of the plant cell.

61. The method of claim 60 wherein the plant cell is grown in culture as a callus.

62. The method of claim 60 wherein the plant cell is grown in culture as a cell suspension.

63. A method of producing a gene product in plant cells, comprising:

harvesting a gene product from a tissue of a plant, said tissue selected from the group consisting of: crown galls, crowns of stem, and axillary buds; said plant, comprising a gene coding for a gene product or for a protein which makes a gene product linked to A. tumefaciens mas 1',2' dual promoter such that mRNA transcribed from the gene initiates at the mas 1',2' dual promoter.

64. The method of claim 63 wherein the axillary buds are collected after a shoot apex has been removed from the plant or after the plant has flowered.

65. The method of claim 63 wherein the tissue is a crown gall which is formed by infecting the plant with A. tumefaciens.

66. The method of claim 65 wherein prior to harvesting the gene product, cells of the crown gall are grown axenically in culture.

67. A method of producing a gene product in plant cells, comprising:

growing a transformed plant cell in culture, said plant cell comprising: a gene coding for a gene product or for a protein which makes a gene product, linked to A. tumefaciens mas 1',2' dual promoter such that mRNA transcribed from the gene initiates at the mas 1',2' dual promoter; and T-DNA of A. tumefaciens which encodes the synthesis of auxin; and

harvesting the gene product from the transformed plant cell or its progeny.

AMENDED CLAIMS

[received by the International Bureau on 21 September 1990 (21.09.90), original claims 1, 5, 9, 44, 45, 60, 63 and 67 amended; other claims unchanged (5 pages)]

1. A method of enhancing the production of a gene product in plant cells, comprising:

linking a gene coding for a gene product or for a protein which makes a gene product to A. tumefaciens mas 1',2' dual promoter to form an expression construct such that mRNA transcribed from the gene initiates at the mas 1',2' dual promoter;

introducing the expression construct into a plant cell;

growing progeny of the plant cell in the presence of about 10 to about 40 uM auxin; and

harvesting the gene product from the progeny of the plant cell.

2. The method of claim 1 wherein the plant cell is grown in culture as a callus.

3. The method of claim 1 wherein the plant cell is grown in culture as a cell suspension.

4. The method of claim 1 wherein after introducing the expression construct into a plant cell, the plant cell is regenerated into a plant, and wherein the progeny of the plant cell growing in the presence of about 10 to about 40 uM auxin is derived from tissue of the plant.

5. A method of enhancing the production of a gene product in plant cells comprising:

linking a gene coding for a gene product or for a protein which makes a gene product to A. tumefaciens mas 1',2' dual promoter to form an expression construct such that mRNA transcribed from the gene initiates at the mas 1',2' dual promoter;

introducing the expression construct into a plant cell;

regenerating the plant cell to form a plant;

harvesting the gene product from a tissue selected from the group consisting of: crown galls, crowns of stem, and axillary buds.

6. The method of claim 5 wherein the axillary buds are collected after a shoot apex has been removed from the plant or after the plant has flowered.

7. The method of claim 5 wherein the tissue is a crown gall which is formed by infecting the plant with A. tumefaciens.

8. The method of claim 7 wherein prior to harvesting the gene product, cells of the crown gall are grown axenically in culture.

9. A method of enhancing the production of a gene product in plant cells, comprising:

linking a gene coding for a gene product or for a protein which makes a gene product to A. tumefaciens mas 1',2' dual promoter to form an expression construct such that mRNA transcribed from the gene initiates at the mas 1',2' dual promoter;

introducing the expression construct into a plant cell;

introducing to the plant cell T-DNA of A. tumefaciens which encodes the synthesis of auxin to form a transformed cell;

growing the transformed plant cell in culture;

harvesting the gene product from the transformed plant cell or its progeny.

10. The method of claim 9 wherein the T-DNA is introduced by electroporation.

11. The method of claim 9 wherein the T-DNA is introduced by transformation.

12. The method of claim 9 wherein the T-DNA is introduced by protoplast fusion of an A. tumefaciens and a plant cell.

13. The method of claim 9 wherein the T-DNA is introduced by infection of the plant cell with A. tumefaciens.

14. The method of claim 1 wherein the expression construct is introduced by electroporation.

15. The method of claim 1 wherein the expression construct is introduced by transformation.

16. The method of claim 1 wherein the expression construct is introduced by infection of the plant cell with A. tumefaciens.

17. The method of claim 5 wherein the expression construct is introduced by electroporation.

18. The method of claim 5 wherein the expression construct is introduced by transformation.

36. The method of claim 34 wherein the reporter gene is a fusion of the lux A and lux B genes of Vibrio harveyi.

37. The method of claim 9 wherein the gene is linked to the mas 1' promoter.

38. The method of claim 9 wherein the gene is linked to the mas 2' promoter.

39. The method of claim 9 wherein a copy of the gene is linked to both the mas 1' and 2' promoters.

40. The method of claim 37 wherein the mas 2' promoter is linked to a reporter gene.

41. The method of claim 38 wherein the mas 1' promoter is linked to a reporter gene.

42. The method of claim 40 wherein the reporter gene is a fusion of the lux A and lux B genes of Vibrio harveyi.

43. The method of claim 41 wherein the reporter gene is a fusion of the lux A and lux B genes of Vibrio harveyi.

44. A plant cell, comprising:

a gene coding for a gene product or for a protein which makes a gene product other than mannopine synthase, linked to A. tumefaciens mas 1',2' dual promoter such that mRNA transcribed from the gene initiates at the mas 1',2' dual promoter; and

T-DNA of A. tumefaciens which encodes the synthesis of auxin.

45. A plant wherein at least some of its cells, comprise:

a gene coding for a gene product or for a protein which makes a gene product other than mannopine synthase linked to A. tumefaciens mas 1',2' dual promoter such that mRNA transcribed from the gene initiates at the mas 1',2' dual promoter; and

T-DNA of A. tumefaciens which encodes the synthesis of auxin.

46. The plant cell of claim 44 wherein the gene is linked to the mas 1' promoter.

47. The plant cell of claim 44 wherein the gene is linked to the mas 2' promoter.

48. The plant cell of claim 44 wherein a copy of the gene is linked to both the mas 1' and 2' promoters.

49. The plant cell of claim 46 wherein the mas 2' promoter is linked to a reporter gene.

50. The plant cell of claim 47 wherein the mas 1' promoter is linked to a reporter gene.

51. The plant cell of claim 49 wherein the reporter gene is a fusion of the lux A and lux B genes of Vibrio harveyi.

52. The plant cell of claim 50 wherein the reporter gene is a fusion of the lux A and lux B genes of Vibrio harveyi.

53. The plant of claim 45 wherein the gene is linked to the mas 1' promoter.

54. The plant of claim 45 wherein the gene is linked to the mas 2' promoter.

55. The plant of claim 45 wherein a copy of the gene is linked to both the mas 1' and 2' promoters.

56. The plant of claim 53 wherein the mas 2' promoter is linked to a reporter gene.

57. The plant of claim 54 wherein the mas 1' promoter is linked to a reporter gene.

58. The plant of claim 56 wherein the reporter gene fusion of the lux A and lux B genes of Vibrio harveyi.

59. The plant of claim 57 wherein the reporter gene is a fusion of the lux A and lux B genes of Vibrio harveyi.

60. A method of enhancing the production of a gene product in plant cells, comprising:

growing a plant cell in the presence of about 10 to about 40 uM auxin, said plant cell comprising a gene coding for a gene product or for a protein which makes a gene product other than mannopine synthase, linked to A. tumefaciens mas 1',2' dual promoter such that mRNA transcribed from the gene initiates at the mas 1',2' dual promoter; and

harvesting the gene product from the progeny of the plant cell.

61. The method of claim 60 wherein the plant cell is grown in culture as a callus.

62. The method of claim 60 wherein the plant cell is grown in culture as a cell suspension.

63. A method of enhancing the production of a gene product in plant cells, comprising:

harvesting a gene product from a tissue of a plant, said tissue selected from the group consisting of: crown galls, crowns of stem, and axillary buds; said plant, comprising a gene coding for a gene product or for a protein which makes a gene product other than mannopine synthase linked to A. tumefaciens mas 1',2' dual promoter such that mRNA transcribed from the gene initiates at the mas 1',2' dual promoter.

64. The method of claim 63 wherein the axillary buds are collected after a shoot apex has been removed from the plant or after the plant has flowered.

65. The method of claim 63 wherein the tissue is a crown gall which is formed by infecting the plant with A. tumefaciens.

66. The method of claim 65 wherein prior to harvesting the gene product, cells of the crown gall are grown axenically in culture.

67. A method of enhancing the production of a gene product in plant cells, comprising:

growing a transformed plant cell in culture, said plant cell comprising: a gene coding for a gene product or for a protein which makes a gene product other than mannopine synthase, linked to A. tumefaciens mas 1',2' dual promoter such that mRNA transcribed from the gene initiates at the mas 1',2' dual promoter; and T-DNA of A. tumefaciens which encodes the synthesis of auxin; and

harvesting the gene product from the transformed plant cell or its progeny.

STATEMENT UNDER ARTICLE 19

Claims 1, 5, 9, 60, and 67 have been amended to specify in the preamble of the independent claims that which is regarded as the invention, i.e., that the claims are directed toward obtaining enhanced expression of genes which are linked to the mas gene promoters in expression constructs.

Claims 44, 45, 60, 63, and 67, which do not include steps of linking genes to the mas promoters and introducing the linked genes to plant cells are amended to specify that the genes which are linked to the mas gene promoters are not the mannopine synthase genes.

FIG. 1

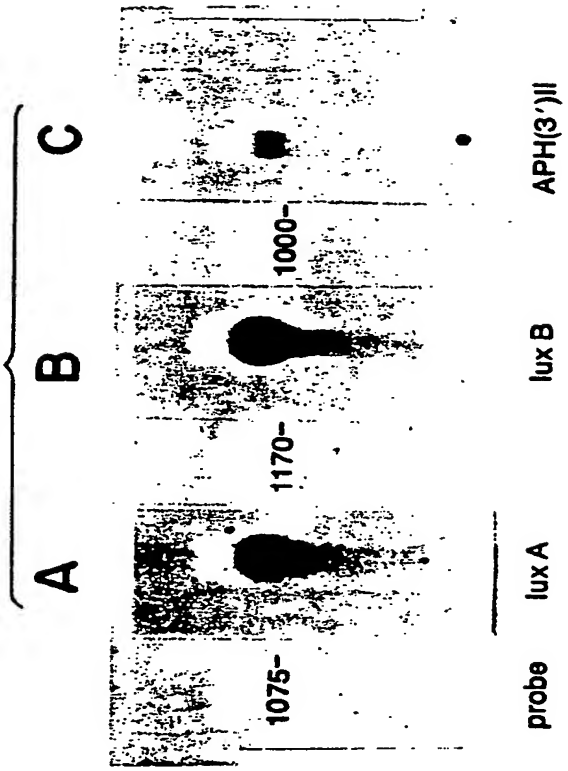


FIG. 2

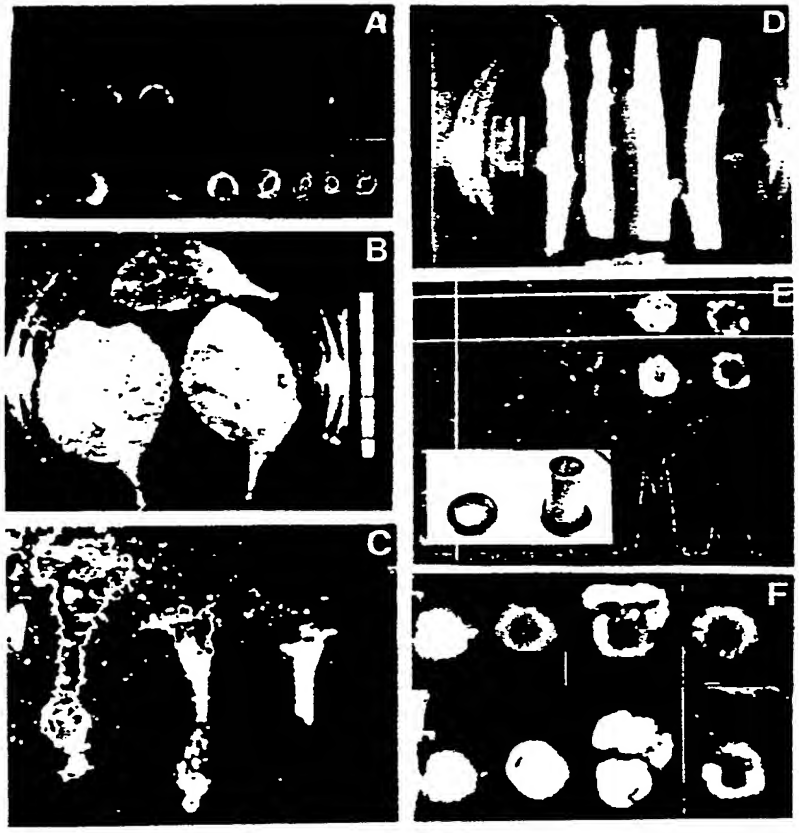


FIG. 3A

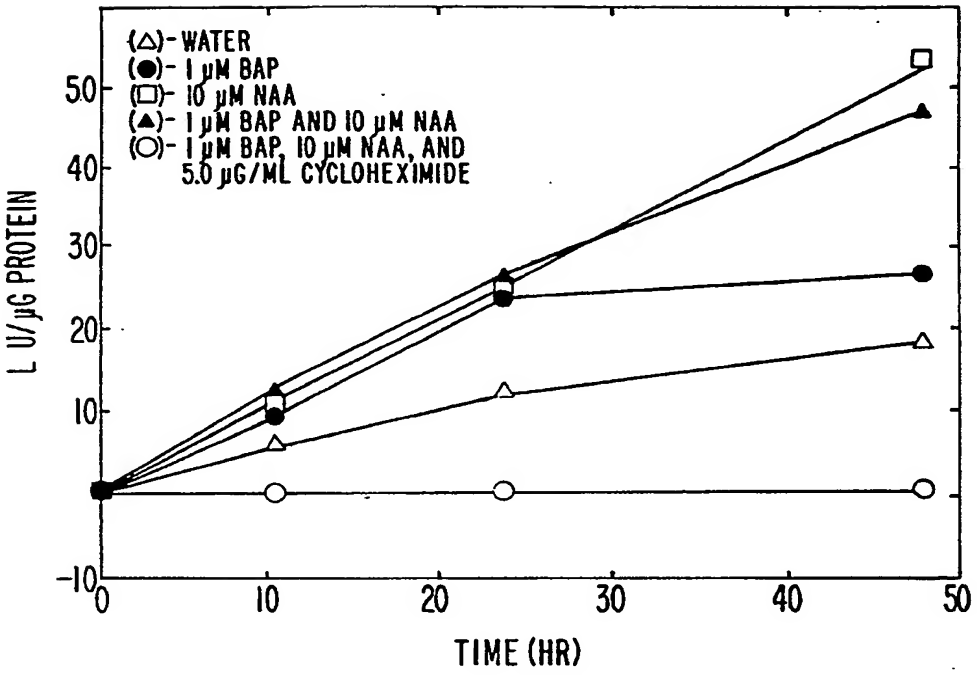
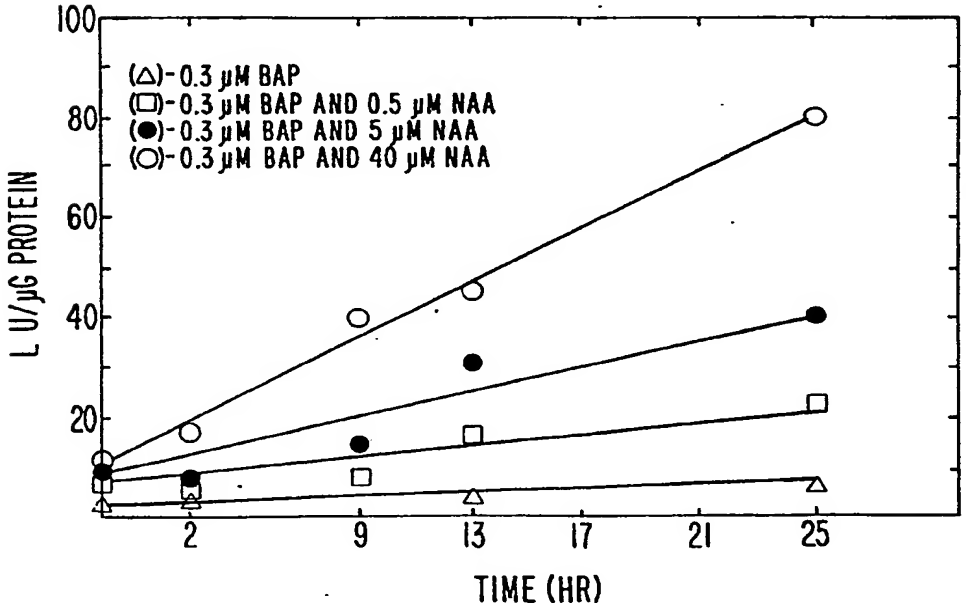

(△)- WATER
(●)- 1 μM BAP
(□)- 10 μM NAA
(▲)- 1 μM BAP AND 10 μM NAA
(○)- 1 μM BAP, 10 μM NAA, AND 5.0 μG/ML CYCLOHEXIMIDE
L U/μG PROTEIN
TIME (HR)

FIG. 3B


(△)- 0.3 μM BAP
(□)- 0.3 μM BAP AND 0.5 μM NAA
(●)- 0.3 μM BAP AND 5 μM NAA
(○)- 0.3 μM BAP AND 40 μM NAA
L U/μG PROTEIN
TIME (HR)

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/01838

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12P 21/00; C12N 15/00; C12N 5/00; A01H 5/00
U.S. CL: 435/69.1, 70.1, 172.3, 240.4; 800/205

II FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/69.1, 70.1, 172.3, 240.4 800/205 935/35,67

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

USPTO AUTOMATED PATENT SYSTEM; DIALOG FILES: BIOTECH
SEE ATTACHMENT FOR SEARCH TERMS

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No [13]
X,P	Proceedings of the National Academy of Sciences USA (Washington DC, USA), Volume 86, Issued May 1989, Langridge et al, "Dual Promoter of Agrobacterium tumefaciens mannopine synthase genes is regulated by plant growth hormones" (see pages 3219-3223).	1-67
X Y	The EMBO Journal (Oxford, England), Volume 3, Issued January 1984, Salomon et al, "Genetic identification of functions of TR-DNA transcripts in octopine crown galls". (see pages 141-146).	1-2,9,11,13, 15-16,21-27, 37-41,44-50, 53-57,60-61, 63, 65-67 1-67
Y,L	Cloning Agricultural Plants Via In Vitro Techniques, CRC Press (Boca, Ratan, FL, USA), Conger (ed.), Conger, "Agronomic Crops". (see pages 172-177, establishes hormone levels of media used in Salomon et al).	1-67
Y	US, A, 4,771,002 (GELVIN) 13 September 1988. See entire document.	1-67
Y	Molecular and General Genetics (Heidelberg, Germany), Volume 204, Issued 1986, Koncz et al, (continue on next sheet)	1-67

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10 MAY 1990	12 JUN 1990
International Searching Authority	Signature of Authorized Officer
RO/US	P. RHODES

Form PCT/ISA/210 (second sheet) (Rev.11-87)

Attachment to FORM PCT/ISA/210, Part II.

II. FIELDS SEARCHED SEARCH TERMS:

Mannopine, mas promoter, dual promoter, opine, cytokinin, auxin, IAA or NAA, transgenic, and phytohormone

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	"The promoter of T_L-DNA gene 5 controls the tissue - specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector". (see pages 383-396).	
Y	Proceedings of the National Academy of Science USA (Washington DC), Volume 84, Issued January 1987, Koncz et al, "Expression and assembly of functional bacterial luciferase in plants". (see pages 131-135).	1-67
Y	The EMBO Journal (Oxford, England), Volume 3, Issued 1984, Velten et al, "Isolation of a dual plant promotor fragment from the Ti Plasmid of Agrobacterium Tumefaciens", (see pages 2723-2730).	1-67
Y	Nucleic Acids Research (Oxford, England), Volume 13, Issued 1985, Velten et al, "Selection - expression plasmid vectors for use in genetic transformation of higher plants", (see pages 6981-6998).	1-67
Y	Molecular and General Genetics (Heidelberg, Germany), Volume 212, Issued 1988, Harpster et al, "Relative strengths of the 35S Cauliflower mosaic virus, 1', 2', and mopaline synthase promoters is transformed tobacco, sugarbeet, and oilseed rape callus tissue", (see pages 182-190).	1-67
Y	Nature (London, England), Volume 328, Issued 1987, Vaeck et al, "Transgenic plants protected from insect attack", (see pages 33-37).	1-67
Y	Developmental Genetics (New York, NY, USA), Volume 8, Issued 1987, Memelink et al, "T-DNA hormone biosynthetic genes: phytohormones and gene expression in plants", (see pages 321-337).	1-67